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Specification as originally filed, with Application for Patent Serial No: **2,209,815**, on July 10, 1997, by **MILLENNIUM INTERNATIONAL CAPITAL INVESTMENTS INC.**, assignee of Andreas Haffner, Frauke Koch, Thomas Kundig, Karoline Zepter and John J. L. Simard, for "Vaccine Method Using Osmotic Pump".

Agent certificateur/Certifying Officer

July 14, 1998
Date

Abstract f the disclosur

A vaccine protocol for inducing long-lasting cytotoxic T lymphocyte (CTL) responses against specific antigen in humans involves use of a controlled release osmotic pump implant. Controlled release drives specific activation of CTL, facilitating continuous recirulation with potent cytotoxic activity for duration of implant.

Osmotic pump vaccine for Cytotoxic T lymphocytes

The present invention relates to a method for vaccination that is particularly relevant for inducing immune responses to tumor cells and other agents that may benefit from potent and prolonged CTL responses. Although used for a variety of purposes, the method involves the use of a kind of system commonly termed controlled release. Various types of controlled release systems exist for delivering pharmaceutical drugs or other pharmacologically active compounds. Microspheres consisting of a polysaccharide matrix have also been used in the development of vaccines (1). The polymer microspheres have several disadvantages as a controlled release system, including residual toxic solvents in the microsphere preparation, less predictable release kinetics, and causing inflammation. These chemical spheres are also limited in terms of the kinds of materials they may contain, and are not suitable for live organisms or virus or for DNA expression systems. Moreover, the system is so far restricted to oral adminsitration to stimulate mucosal antibody (IgA) immunity (2).

The invention described herein relates to a method that offers several major advantages over existing methods used for vaccines designed to stimulate specific cell-mediated (Cytotoxic T lymphocyte) immunity: (a) it provides for the use of a "mechanical antigen depot," circumventing the need for adjuvants that induce antigen depot effect via the induction of granulomatous lesions at the site of vaccine injection; (b) it provides a means for ongoing delivery of antigenic stimulation, a critical requirement for maintaining active CTL responses, (c) it facilitates the induction of CTL immunity exclusively, without promoting TH2-type antibody responses that tend to downregulate the strength of CTL responses; (d) it circumvents the need for immunostimulants, i.e. BCG, attenuated virus, as part of an adjuvant formulation, that pose significant risk of

systemic illness or infection; and (e) it facilitates the efficient and direct induction of CTL using class I restricted peptides, normally unable to induce responses because of their short half-lives in the body.

CTL are white blood cells found in the blood, spleen and lymph. Their function is to destroy (1) virus-infected cells and (2) tumor cells that display tumor-associated antigen. After induction by antigen, i.e. immunization, a CTL precursor cell (CTLp) will divide into daughter cells with the same antigenic specificity (i.e. proliferate), it will become a mature CTL, and will recirculate through the body with the ability to identify and destroy cells bearing the specific antigen. Recent data has shown that an active CTL immune response against a specific antigen requires continuous antigenic stimulation with that antigen (Kundig et al. 1996). In the absence of antigen, once activated CTL soon cease to recirculate through the body and find their way to the spleen. Since CTL must deliver a lethal hit directly, their residence in the spleen precludes an active role in protection against infections or tumor growth at distant sites in the body (Kundig 1996).

CTL play a unique role in protecting against both a number of different pathogens and tumors (4). The failure to induce effective CTL responses against pathogens or tumors can result in chronic disease. Consequently, significant effort has been devoted towards developing vaccines that induce CTL responses against infectious agents or tumors. Despite the efforts, however, methods for inducing "active" CTL immunity have been slow in developing. Although it has been possible to induce transient CTL responses and to increase specific CTLp in the spleen, there has been no method described for inducing long-lived CTL responses (3). Nonetheless, there are situations, particularly in the case of tumors, which are notorious for their lack of immunogenicity, where long-lived CTL responses might have significant therapeutic value.

There is a fundamental reason why researchers have failed to induce long-lived CTL responses. For activation, CTL must be exposed to antigen in the context of MHC molecules on the surface of cells. However, once activated, CTL target the same antigen presenting cells for destruction. This paradoxical situation precludes the continuous antigenic stimulation necessary to maintain active CTL responses.

Potent adjuvants have been used to cause wound (granuloma) formation, which entraps antigen and provides an antigen depot effect (5). In the process of causing granluomas formation, these adjuvants can cause serious lesions and systemic illness, including shock and death. Although the immunostimulatory effect is often regarded as an essential feature of the adjuvant, it is non-specific, typically resulting in the induction of so called TH2-type responses that interfere with strong CTL immunity. In addition to their toxicity, adjuvants have thus proved very inefficient at inducing strong and long lasting CTL responses.

Genetically engineered viruses modified to carry a gene encoding a specific antigen have been used as a vehicle for immunization. However, this method is intrinsically flawed, since clearance of the virus infection results in clearance of the antigen. The CTL inducing effect is therefore transient. Repeated injection with virus has been attempted, but this results in strong antibody responses against the virus, accelerating the speed by which the virus is cleared and further reducing the efficacy of the system.

DNA vaccines are also being developed for the purpose of inducing CTL immunity. Once again, the system has intrinsic limitations that preclude its efficacy in inducing long-lasting CTL immunity. The DNA vaccines consist of a plasmid or similar genetic construct for expressing the antigen of interest. Up-take of the plasmid by cells of the body results in expression of the antigen and induction of CTL. As above, however, once cells expressing the construct have succeeded in inducing CTL, they are themselves targets for eradication by the CTL. The CTL inducing effect is thus again transient.

Potent tumor specific CTL responses can be induced by vaccines. However, studies have shown that once the active CTL response has subsided, i.e. CTL have stopped recirculating and have taken occupancy in the spleen, tumor cells can grow unopposed in the body (Unpublished results). Thus it is clearly critical for complete eradication and protection against resurgent tumor, that active CTL responses are maintained for an extended period.

The "osmotic pump" controlled release device offers a means by which to provide ongoing antigenic stimulation. It is an inert mechanical device made of titanium and is hypoallergenic. Thus it causes no significant irritation. Unlike adjuvants,

which create an antigen depot through stimulating granuloma formation, the osmotic pump acts directly as a mechanical antigen depot. The device cannot be destroyed by CTL. Thus it goes on working to deliver antigen for the duration it is implanted in the skin (Figure 1). Importantly, our recent findings have shown that the immunostimulatory effects of adjuvants may not be needed when constant antigenic stimulation is present (6). Additionally, if antigen stimulation can be directed to the appropriate environment, i.e. lymphoid organ, immunostimulants or costimulation can be circumvented (7); such control over antigen delivery is feasible using a mechanical pump devivce for vaccine delivery. Finally, since conventional adjuvants are not required, i.e. to induce granuloma formation or immunostimulation, the osmotic pump device can be used to deliver only CTL-inducing formulations rather than those that enhance competing TH2-type immune responses (8).

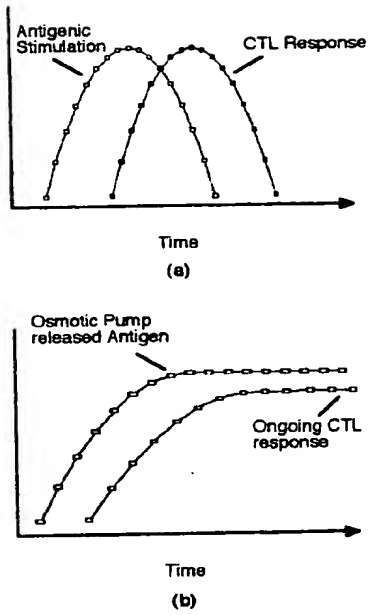

Figure 1. CTL response to Antigen
(a) Vaccines can only deliver specific antigen for a short time before the antigen is cleared by the body. Kinetics of the CTL response resembles the residual antigen profile.
(b) Continuous delivery, whether involving an expression system such as a DNA construct or a recombinant virus or organism delivering the target antigen, facilitates an ongoing CTL response for the duration of implant of the delivery system. (Residual antigen: black line Active CTL response: red line)

~~**Embodiments of the Invention for Which an Exclusive Property or Priviledge is Claimed are defined as follows:**~~

~~1. Use of controlled release implantable "osmotic pump" device for continuous delivery of antigen or antigen expression systems, such as DNA constructs including plasmids, recombinant viruses, and recombinant organisms.~~

~~2. Said delivery as claimed in 1, to provide long-lasting antigenic stimulation and ongoing recirculation of CTL. The CTL response can be maintained for a period of 12 months compared to current methods that induce response for only about 2 weeks.~~

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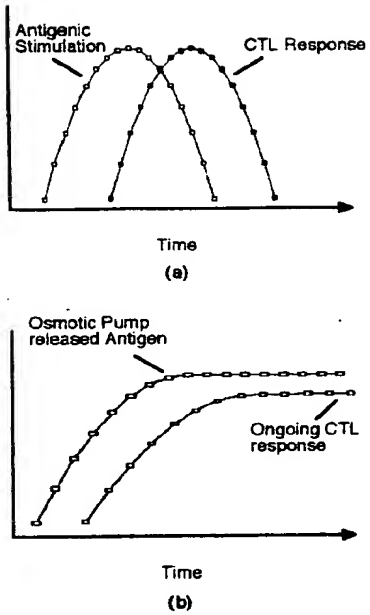

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2. Said delivery as claimed in 1, to provide long-lasting antigenic stimulation and ongoing recirculation of CTL. The CTL response can be maintained for a period of 12 months compared to current methods that induce response for only about 2 weeks.

3. Said delivery as claimed in 1, as a mechanical antigen depot. This replaces need for adjuvants that cause indirect antigen depot through stimulating body to induce granulomatous lesions at site of vaccine injection.

4. Said delivery as claimed in 1, acts as "constant injector," avoiding the need for repeated injection of expression-type vaccines, such as DNA constructs or recombinant virus designed to express target antigen, in order to increase the frequency of antigen-specific CTL.

5. Said delivery as claimed in 1, acts as a mechanical adjuvant that provides constant CTL immunostimulation, circumventing the need for highly toxic adjuvants used as immunostimulants.

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B&P File No. 10068-003/MG

Title: A Method of Inducing a CTL Response

FIELD OF THE INVENTION

The invention relates to a method of inducing a CTL response to an antigen by delivering the antigen directly into the lymphatic system using a controlled release delivery vehicle.

BACKGROUND OF THE INVENTION

Cytotoxic T lymphocytes (CTL) are white blood cells found in the blood, spleen and lymph. CTL have the ability to attack and kill other cells of the body in a highly specific manner. When CTL are stimulated by specific antigen, they migrate through the tissues of the body on a "search and destroy" mission for cells bearing the specific antigen. Whether of viral origin or tumor associated, CTL detect antigen that is bound to major histocompatability complexes (MHC) on the surface of potential target cells. Once CTL have identified the antigen on the cell surface, their function is to deliver a lethal hit to the cell.

Although there are hundreds of millions of CTL that reside in the spleen, each individual CTL exclusively responds to a unique and specific antigen. These individual CTL, dubbed CTL precursors (CTLp), undergo cell division or proliferate upon activation by specific antigen to produce daughter cells with precisely the same antigen specificity as the parent cell. This proliferation increases the total number, and thus the frequency, of that specific CTLp in the body. A proportion of these newly generated CTL briefly recirculate through the body (termed effector CTL), and have the ability to identify and destroy cells bearing the specific antigen which they recognize. A significant body of experimental evidence has now shown that CTL specific for tumor antigens can indeed prevent or eradicate tumors (1-6). Unfortunately, most tumors have only a very weak capacity to stimulate CTL responses (7-9); and the many attempts to directly enhance the immunogenicity of tumor cells, such that they might directly stimulate

tumor-clearing CTL responses in patients has met with limited success (10). Technical advances over the past ten years have, however, enabled the identification of natural peptide antigens that are present on tumor cells and which are recognized by CTL. These antigen targets include proteins expressed in significant overabundance, abnormally expressed embryonic proteins, protein products from mutated oncogenes or suppressor genes, or proteins derived from cancer-causing viruses present in tumor cells (11). The challenge has been to find a way in which to administer these antigens in a vaccine formulation, such that it is effective at inducing anti-tumor CTL reponses. While many attempts have now been made to use these antigens in a clinical setting, the results have been less than satisfactory (12).

Recent key observations made by the inventor and others may explain why CTL therapies have been largely ineffective at eradicating or controlling tumors in a clinical setting. These observations include the following:

(a) Vaccine designs have been inadequate at initiating strong CTL responses (12);

(b) Tumor cells can down regulate MHC molecules, resulting in the loss of antigen presentation from the surface of cells, thereby escaping detection by CTL (13-19);

(c) After induction, effector CTL recirculation through the body is highly transient (20);

(d) Active CTL immunity is dependent on continuous immunogenic stimulation by antigen;

(e) After recirculation, CTL return to the spleen where they reside in a non-active or resting state (20); As a corollary of (e), an increase in the numbers of CTLp residing in the spleen does not reflect active CTL immunity (20,21);

(f) In the case of tumors, regrowth of residual tumor cells following immunization goes undetected by CTLp residing in spleen in a "resting" state (21).

A growing repertoire of tumor associated antigens are being discovered that are recognized by CTL (22-24). A variety of techniques have been suggested to render these antigens effective in CTL vaccines. These include immunization using synthetic peptide antigens mixed with an immunostimulatory adjuvant, such as the bacterial toxin BCG; immunization with multiple antigenic peptide systems (MAPS) (25-27); immunization with "professional" antigen presenting cells, which are isolated from the patient, pulsed with peptide antigen and innoculated back into the patient as a vaccine (28-29); immunization with peptides designed to stimulate both CTL and T helper cell populations (30); immunization with viruses or bacteria engineered to express tumor antigens (31-33); and immunization with polynucleotide expression vectors (so called DNA vaccines) (34). Unfortunately, none of these approaches have been an unqualified success. As discussed above, the lack of vigorous therapeutic effects with these vaccine platforms reflects at least to some degree problems associated with inducing a strong initial CTL response and with maintaining ongoing "active" CTL immunity.

Studies by Glenny during the first quarter of the century revealed that aluminum compounds could enhance the strength of diptheria vaccines (35). This was ostensibly the first of long history of observations supporting a "depot" theory of immunization, which postulates that antigen slowly leaking into the tissues over an extended time corelates with the antigenic potency of a vaccine. Today, this antigen depot paradigm forms the intellectual backdrop to most non-cytokine-based adjuvant development programs. In one form or another, depot type adjuvants are intended to prolong the course of antigen delivery, by forming a lesion at the site of injection, or simply by the slow degradability of the adjuvant itself, which mixed with the specific antigen forms a depot at the site of injection. A second function generally attributed to adjuvants are their immunostimulatory effects, which appears to trigger the immune system to respond to the vaccine.

However, adjuvants are a double-edged sword. They have inherent toxicities. But it is a feature of these toxicities that achieves a desired immunostimulatory and/or depot effect. Side effects such as tissue damage and granulomatous reaction at the site of injection, fever, and in some cases sytemic reactions, such as Reiter's syndrome-like symptoms, uveitis and arthritis are the some of the risks associated with the use of adjuvants (36-37). Currently, the only adjuvant approved by the FDA is alum. It is relatively safe but does have side effects such as erythema, subcutaneous nodules, contact hypersensistivity, and granulomatous inflammation (38). More importantly, alum only acts to potentiate a limited number of antigens, and it very predominantly stimulates humoral (Th2) resonses rather than CTL immunity (39). Thus so far adjuvants have proved to be very ineffective components for vaccines aimed at inducing clinically relevant CTL responses.

Recent attempts to induce CTL responses using dendritic cells or other antigen presenting cells, despite being cumbersome, have shown some promise (40-41). New recombinant virus or bacterial systems carrying genes for specific antigen are effective at inducing primary CTL responses (42). The most effective viruses, for example, that induce strong CTL responses are those (perhaps not surprisingly) which replicate aggressively in the host. Yet because of the risk for serious or lethal complications as a result of infection, recombinant organisms used in a cancer vaccine must be only weakly replicative, or be completely replication deficient. This trade-off between virulence and efficacy is at present an intractable problem (43).

Nonetheless, based on the inventor's recent elucidation of the nature of CTL immunity, methods that involve single delivery of vaccines are inherently limited to inducing only transient CTL immunity. This reflects the observation by the inventor that active CTL immunity requires persisting antigenic stimulation (20). Moreover, since APC are targeted for destruction by the same CTL that they have activated, the CTL response is self-limiting. This self-limiting aspect of

CTL immunity precludes, under normal circumstances, the continuous stimulation necessary to maintain long-lived CTL responses. While this makes evolutionary sense for defense against natural infection, where CTL remain active only as long as the infectious agent replicates and persists in the host, in terms of a tumor vaccine, which must support the CTL response in lieu of immunogenicity of tumor cells themselves, the self-limiting nature of CTL immunity must be seen as a central concern in the design of a CTL vaccine (21).

DNA (or polynucleotide) vaccines are also being developed for the purpose of inducing CTL immunity (24,44). Once again, the system has intrinsic limitations that preclude its efficacy in inducing long-lasting CTL immunity. The DNA vaccines consist of a plasmid or similar genetic construct for expressing the antigen of interest. Uptake of the plasmid system by cells of the body results in expression of the antigen and induction of CTL. However, once cells expressing the construct have succeeded in inducing CTL, they are themselves targets for eradication by the CTL. The CTL inducing effect is thus again transient. Moreover, the polynucleotide vaccines have thus far suffered from poor efficiency in terms of CTL induction.

With difficulties in achieving strong primary and/or persisting CTL responses, there are a number of clinical trial groups now using repeated injections of cancer vaccines. The use of antigenically complex materials in the vaccine formulation, such as recombinant virus, or the costs associated with repetitive treatment using cultured APC will, however, make such an approach difficult. On the one hand, repetitive immunization with antigenically complex materials drives the immune system to elaborate a humoral, as opposed to a CTL, response (45). While on the other hand, use of a minimal CTL antigen (such as a nonamer peptide) which does not efficiently drive a humoral response, also fails to induce a CTL response. Attempts to develop adjuvants that enhance the immunostimulatory aspects of minimal CTL antigens have resulted in the production of materials (i.e.

adjuvants) that also induce a competing humoral immune response, or, which simply offer little CTL stimulatory effect.

As can be seen from the foregoing, there has been little success at developing a CTL vaccine that is both capable of inducing, and maintaining, CTL immunity. The development of a CTL vaccine with these capabilities is essential before effective anti-tumor therapy based on CTL immunity can be contemplated.

SUMMARY OF THE INVENTION

The present inventor has developed a method for inducing a potent and prolonged CTL response to an antigen, which overcomes the drawbacks of the prior art methods.

Broadly stated, the present invention provides a method of inducing a CTL response to an antigen comprising delivering the antigen into the lymphatic system of an animal. In a preferred embodiment, the antigen is delivered into a lymphatic organ, such as a lymph node.

The antigen may be delivered by a variety of methods including direct injection into the lymphatic system, by an antigen delivery vehicle that is implanted at or near a lymphatic organ or by an antigen delivery vehicle that is external to the animal but contains a means to deliver the antigen into the lymphatic system. The antigen is preferably delivered continuously into the lymphatic system for a predetermined period of time. The antigen may be delivered using a controlled release delivery vehicle. In one embodiment, the antigen delivery vehicle is an osmotic pump that can effect the controlled, continuous delivery of antigen directly into a lymphatic organ, such as a lymph node.

The method of controlled release into a lymphatic organ resolves several fundamental limitations with prior art: (A) it facilitates the induction of CTL immunity exclusively, without promoting TH2-type antibody responses that tend to downregulate the strength of CTL responses; (b) it circumvents the need for toxic and or infectious

vehicles as part of an adjuvant or carrier, respectively, that pose significant health risk; (c) it facilitates the efficient and direct induction of CTL using engineered class I restricted antigens, normally unable to induce responses; and (d) it allows for continuous antigen stimulation, which is essential to maintain active CTL immunity.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1 is a graph showing the lysis of target cells by CTL versus the effector/target ratio when antigen is delivered as a single dose (circles) and when antigen is delivered by a continuous pump (triangles).

Figure 2 (A and B) are graphs showing the lysis of target cells by CTL versus the effector/target ratio when antigen is delivered as a single dose (circles), when antigen is delivered by a continuous pump (triangles) and negative control (squares) at (A) 36 hours and (B) 7 days.

Figure 2C is a graph showing the footpad swelling versus time when antigen is delivered as a single dose (circles) and when antigen is delivered by a continuous pump (triangles).

Figure 3 is a graph showing the lysis of target cells by CTL versus the dose of the peptide antigen when the antigen is delivered subcutaneously, intravenously and intrasplenically.

Figure 4 is a bar graph showing tritiated thymidine uptake in CTL cells induced by antigen introduced intravenously, intrasplenically and subcutaneously.

DETAILED DESCRIPTION OF THE INVENTION

As hereinbefore mentioned, the present invention relates to a method of inducing a CTL response to an antigen comprising delivering the antigen into the lymphatic system of an animal. The term "animal" as used herein means any member of the animal kingdom capable of having a CTL response, and includes all mammals such as humans. The antigen may be delivered by a variety of methods including direct injection into the lymphatic system, by an antigen delivery vehicle that is implanted at or near a lymphatic organ or by an antigen delivery vehicle that is external to the animal but contains a means to deliver the antigen into the lymphatic system.

The antigen is delivered continuously into the lymphatic system for a pre-determined amount of time. The antigen can be delivered for as long as a CTL response is desired and can range anywhere from hours to days to months and even years, if necessary. The antigen is preferably administered in a controlled release fashion, more preferably using a controlled release delivery vehicle. The present inventor has determined that controlled release of antigen into the lymphatic system induces and sustains the CTL response to the antigen. The antigen is preferably delivered into a lymphatic organ, such as a lymph node.

In one embodiment, the antigen is delivered to the lymphatic system through an osmotic pump that is implanted into the animal at or near a site of a lymphatic organ. The osmotic pump can be any pump that can deliver the antigen at a controlled rate over a pre-determined period of time and is suitable for use in the host. Several pumps are known in the art for the delivery of agents (such as drugs) in humans and these can be used or adapted for use in the present invention.

A basic osmotic pump incorporates a cylindrical housing containing a chamber for storing the material to be delivered, separated from a compartment containing an osmotic salt material by a barrier that is moveable under pressure such as a piston or a flexible impermeable membrane. The compartment containing the osmotic salt is separated from osmotic fluid by a semipermeable membrane. In some embodiments, a fluid barrier, such as a foil sheet, isolates the osmotic salt chamber from the osmotic fluid, keeping the pump inactivated until removal of the barrier immediately prior to use. Other osmotic pump devices use body fluid as the osmotic fluid. In these devices a semipermeable membrane separates the osmotic salt compartment from body fluids, and the pump is activated once inserted into the body under exposure to body fluids. In either case, volumetric expansion of the osmotic salt drives the expulsion of the stored material from the compartment and into the surrounding environment of the body. These pumps have been highly successful at achieving steady-state pumping and delivery of agents. The pumps are of a small size that can be inserted into a patient, with flexible consideration as to location. This is important in the case of CTL vaccines, since the inventor has determined that efficient induction of CTL responses is contingent on the antigen or antigen expression system being delivered into the lymphatic system, in order to ultimately achieve antigen delivery into a primary lymphatic organ such as the spleen. The present inventor has determined that antigen delivered into a lymph node is one-million times more efficient at inducing CTL responses compared with conventional subcutaneous delivery. A modification to the osmotic pump incorporates a microcatheter attachment at its discharge end, such that when the pump is implanted proximal to a lymphatic organ, such as a lymph node, the catheter can be inserted into the organ to facilitate delivery of the vaccine directly into the lymphatic system.

In a further embodiment, the vaccine is delivered using an external pumping mechanism, that is directly discharging into the

lymphatic system through cannulation of a lymphatic vessel such as a lymph node. The external pump may be an insulin-type pump, that has been loaded with the appropriate vaccine formulation. In a specific embodiment, an insulin-type pump can be used to deliver a CTL vaccine via a small tube that is cannulated into a lymphatic vessel such as the inguinal lymph nodes, which are appropriately large and thus well suited for catherization. In all cases where catheterization of the lymphatic vessel is involved, ultrasound positioning can be used to accurately position the catheter into the lymph vessel. Regular follow up ultrasound evaluations should be performed to ensure catheter placement remains intact, which is important to the function of the vaccine.

In another embodiment, the antigen is incorporated into biodegradable vehicles that are implanted or injected into the lymphatic system or organ. In one specific embodiment, the biodegradable vehicle is a microsphere containing the desired antigen. The use of microspheres made from polylactic glycolic acid (PLGA) has been reported for a number of different applications including vaccines (46-50). The PLGA particles are suitable for controlled release vehicles, since they entrap material in a polymer matrix that has predictable degradation rates that, depending on the exact formulation, range from a few weeks to as much as one year. The material also has very good biocompatability. The microspheres degrade in vivo into the normal metabolites, lactic and glycolic acids, releasing entrapped contents into the surrounding medium. A double/emulsion/solvent evaporation technique can be used to prepare microspheres that entrap vaccine formulations, including materials such as proteins or peptide antigens and potentially other biological or recombinant antigens or antigen expressing systems such as naked DNA plasmids. These vaccine components, designed to drive specific CTL reponses, would be released upon degradation of the microspheres. It is the finding of the inventor that release of such vaccine components into a lymphatic organ would

facilitate strong induction of CTL immunity (75). The time-release or controlled release aspect of the delivery system in the lymphatic organ would also facilitate long term provision of antigen, which is absolutely essential for maintaining active CTL immunity (20, 21).

In another specific embodiment, the biodegradable vehicle is a liposome containing the desired antigen. Liposomes were first described in 1965, produced from phospholipids that formed artificial lipid vesicles in water and resembling natural biological membranes (51). Liposomes have found a use in vaccines (52-53), where liposomes probably function to direct delivery of antigen into antigen presenting cells such as macrophages (54) and dendritic cells (55), which in turn facilitates induction of CTL (56-57).

Prior to the administration of the antigen using any of the above vehicles, methods may be used to assist in the determination of the optimum location for the antigen delivery. For example, when using the osmotic pump, radiography may be used to image a patient's lymphatic flow, to determine where relatively high lymphatic drainage occurs, in order to decide upon an insertion position for the osmotic pump that maximizes delivery into the lymphatic system. Since each patient has unique lymphatic drainage profiles, imaging would be conducted for each individual prior to insertion of osmotic pump for delivery of antigen. When using direct cannulation of the lymphatic vessel, such as in the use of osmotic or insulin pumps to deliver antigen, ultrasound can be used to position the needle directly into the lymphatic vessel and to monitor its positioning over the period of treatment.

The inventor has found that antigen delivery into a lymphatic organ, such as a lymph node, results in antigen-loading of dendritic cells, which are potent antigen presenting cells (APC). The inventor has thus demonstrated that the controlled delivery of antigen into a lymphatic organ causes potent CTL induction through efficient *in vivo* loading of dendritic cells. There are several advantages to loading

the antigen *in vivo* rather than *in vitro* as in the prior art methods which include [a] it facilitates loading of dendritic cells without the time-comsuming and technically demanding task of removal of cells from the patient and culture in the laboratory; [b] it does not suffer from the losses of dendritic cells that occurs upon transfer from patient to culture and from culture back into patient; [c] the effect of vaccination is immediate upon the delivery of antigen into the lymphatic organ; [d] CTL induction is not transient, since stimulating antigen can be maintained continuously for the duration of delivery; and [e] long-term induction of CTL is expected to have significant therapeutic benefit to patients.

Antigen used in the present invention can be any antigen that induces a CTL response. In a preferred embodiment the antigen is a tumor antigen and the method of the present invention can be used in tumor immunotherapy. Examples of tumor antigens that may be used include: differentiation antigens such as MART-1/MelanA (MART-1), gp100 (Pmel 17), tyrosinase, TRP-1, TRP-2 and tumor-specific multilineage antigens such as MAGE-1, MAGE-3, BAGE, GAGE-1, GAGE-2, p15 (58); overexpressed embryonic antigens such as CEA; overexpressed oncogenes and mutated tumor-suppressor genes such as p53, Ras, HER-2/neu (59); unique tumor antigens resulting chromosomal translocations such as BCR-ABL, E2A-PRL, H4-RET, IGH-IGK, MYL-RAR (58); and viral antigens, such as the Epstein Barr virus antigens EBVA (60) and the human papillomavirus (HPV) antigens E6 and E7 (61).

Antigens may be used alone or may be delivered in combination with other compounds including GM-CSF, IL-12, IL-2, TNF and IFNγ that are known to enhance immune stimulation of CTL reponses. Many animal and human tumors have been shown to produce cytokines such as IL-4 (62), IL-10 (63-65), TGF-β (66-67) that are potent modulators of the immune response that protect tumors from immune-mediated destruction (68). The production of IL-4, Il-10 or

TGF-β by the tumors may achieve this protective effect by suppressing the induction of cellular immunity, including the elaboration of CTL responses (68). Alternatively, cytokines that support CTL responses can be exogenously added to help in the tug-o-war between induction of anti-tumor cell mediated and non-tumor-destructive humoral responses. The present inventor has used several such exogenous cytokines in experimental mouse vaccination models which are known to enhance CTL responses, including GM-CSF, IFNγ, IL-2. An effective exogenous cytokine that may be used is GM-CSF. GM-CSF is reported to enhance the expression of the so called "co-stimulatory" molecules B7-1 and B7-2 on antigen presenting cells (APC) (69), which are important players in the symphony of interactions that occur during stimulation of CTL by APC. Moreover, GM-CSF is known to induce activation of APC and to facilitate growth and differentiaion of APC (70), thereby making these important CTL stimulating cells available in both in greater numbers and potency (71-72). GM-CSF has also been reported elsewhere to potentiate anti-tumor CTL responses (73). Other cytokines known to stimulate CTL, such as IL-12, may also be useful in the antigen vaccines of the invention.

The method of the present invention is clearly advantageous over the prior art methods for inducing a CTL response against a tumor. For example, the present invention does not require repetitive immunizations to effect for prolonged anti-tumor immunotherapy. The continuous delivery of the antigen maintains the CTL response that could ultimately afford a prolonged aggressive posture of CTL against tumor cells, more thorough eradication, and protection against reoccurrence during the vaccine treatment. In the absence of antigen, CTL that have undergone primary activation soon cease to recirculate through the body, soon finding their way to the spleen where they become quiescent. Since CTL must deliver a lethal hit directly, their residence in the spleen precludes an active role in protection against infections or tumor growth at distant sites in the body.

The controlled release of CTL antigen circumvents this outcome as antigen delivery is maintained. Controlled released antigen delivery to a lymphoid organ this solves two major problems: It provides for potent CTL stimulation that takes place in the milieu of the lymphoid organ (74); and it provides continuous stimulation that is necessary to keep CTL active, cytotoxic and recirculating through the body (20,75).

Another fundamental improvement of the present method over prior art is that it facilitates the use of inherently non-immunogenic peptide antigens for CTL stimulation without the combined use of conventional adjuvants. This is very beneficial as most experimental adjuvants are toxic and poorly suited for use in humans. In addition adjuvants stimulate the TH2-type humoral immune response that negatively affects the CTL response. Further, since conventional adjuvants are not required, only the minimal antigenic epitope for a CTL response is required in the formulation.

An additional advantage to the method of the present invention, where it embodies the use of mechanical delivery systems, is that the antigen delivery can be stopped if any adverse immunological effects are observed. For example, in vaccines against melanoma, CTL have been induce to attack not only malignant melanocytes but also healthy tissue, causing "vitiligo" (76). The ability to discontinue a CTL vaccine at any time is a significant advance in vaccine safety.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

Materials and Methods

Mice: The generation of T cell receptor transgenic mice (TCR+ mice) in which approx. 90% of the CD8+ T cells express a TCR recognizing the immunodominant LCMV-glycoprotein epitope (gp-peptide aa33-41, p33) presented on H-2Db, has been described in detail (75). All experimental mice were between 8 and 12 weeks of age and bred and held under strict

pathogen free conditions at the Institut Für Laborrtineikunde at the University of Zurich.

Viruses: LCMV (Armstrong strain) was originally obtained from Dr. M.B.A. Oldstone, Scripps Clinics and Research Foundation, LaJolla, San Diego, CA. (76). Seed virus was grown on BHK cells and plaqued on MC57 cells using an immunological focus assay, as described previously (77).

Osmotic pump: ALZA model #1007b.

In vivo protection assays for specific CTL activity: The *in vivo* assay for the detection of CTL activity by challenge infections with LCMV has been described in detail previously (Oehen et al. 1991). Briefly, mice are intravenously challenged with 2X103 pfu of LCMV (Armstrong). After 4 days the titer of LCMV is determined using the above mentioned immunological focus assay (77).

Primary ex vivo cytotoxicity against LCMV-gp: Mice were injected intravenously with 10µg of p33. After 36 hours spleen single cell suspensions were coincubated for 5h with 51Cr-labeled syngeneic EL-4 (H-2b) target cells, that were either pulsed with p33 or left unpulsed. Specific lysis was calculated as [(experimental 51Cr release - spontaneous 51Cr release) / (total 51Cr release -spontaneous 51Cr release) X 100%].

LCMV induced foot pad swelling reaction: Mice were infected with LCMV (Armstrong) by intradermal injection into the hind footpad (5000 pfu in 30µl). Footpad thickness was measured daily with a spring caliper. Footpad swelling is calculated as (measured thickness - thickness before injection) / (thickness before injection).

Example 1

Continuous release of peptide antigen using osmotic pump induces potent CTL response in C57BL/6 Mice

C57BL/6 mice were either intravenously injected with a single dose of 50µg p33 (including 500 ng GM-CSF) or were implanted with a microsomotic pump releasing a mixture of 50µg of p33 and 500 ng GM-CSF over a time period of 7 days, or were left naive. After 7 days

mice were sacrificed to prepare single cell suspensions from the spleen. Spleen cells were restimulated *in vitro* for 7 days in the presence of low amounts of IL-2 and p33 peptide according to the method of Kündig et al (20). Specific cytotoxicity was measured using ^{51}Cr-labeled EL-4 target cells pulsed with p33. Specific lysis of EL-4 target cells without p33 was less than 16% for all effectors. The results are shown in Figure 1.

Example 2

Continuous release of antigen induces CTL immunity against virus in C57BL/6 mice

C57BL/6 mice were either intravenously injected with a single dose of 50µg p33 (including 500 ng GM-CSF. Pharmingen) or were implanted with a microsomotic pump releasing a mixture of 50µg of p33 and 500 ng GM-CSF over a time period of 7 days, or were left naive. After 7 days specific CTL activity was assessed *in vivo* using anti-viral protection assays. C57BL/6 mice were intravenously challenged with LCMV Armstrong strain ($2x10^3$ p.f.u.). After 4 days mice were sacrificed and LCMV titers were determined in spleens using an immunological focus assay (77). Mice implanted with osmotic pump showed significantly lower virus titers indicating active CTL immunity against the virus (Table 1).

Example 3

Continuous release of antigen maintains potent CTL effectors in TCR Transgenic Mice

TCR transgenic mice were either intravenously injected with a single dose of 50µg p33 or were implanted with a microsomotic pump releasing a mixture of 50µg of p33. After 36 hours mice were sacrificed to prepare single cell suspensions from the spleen which were assayed for ex vivo p33-specific cytotoxicity using ^{51}Cr-labeled EL-4 target cells pulsed with p33. Similarly mice were either intravenously injected with a single dose of 50µg p33 or were implanted with a microsomotic pump releasing a mixture of 50µg of p33 over a time period of 7 days, or were left naive. After 7 days mice were sacrificed to prepare single cell

suspensions from the spleen to assay ex vivo p33-specific cytotoxicity using ^{51}Cr-labeled EL-4 target cells pulsed with p33. Specific lysis of EL-4 target cells without p33 was less than 18% for all effectors. The results are shown in Figures 2A and 2B.

Continuous release of maintains protective CTL response against virus infection

After 7 days mice TCR transgenic mice were challenged by intradermal LCMV injection into their hind foot pads (2x103 pfu in 30µl). The absence of a foot pad swelling reaction, as observed in mice with an implanted pump, indicates that at the time point of injection there was active CTL immunity inhibiting local replication of the virus in the foot pad. In contrast, foot pad swelling, as observed in mice injected the peptide as a single bolus and naive control mice, indicated that LCMV successfully replicated in the foot pad in the absence of protective CTL. The results are shown in Figure 2C.

Example 4

Direct delivery of antigen into lymphatic organ dramatically increases efficiency of CTL induction

TCR transgenic mice were injected with graded doses of gp-peptide p33 either subcutaneously (S.C.), intravenously (I.V.) or directly into the spleen (I.S.) via a small abdominal incision. The efficiency of CTL induction was assessed by measuring gp-specific CTL activity 24 hours after injection. CTL activity is known to peak on day after injection of peptide. Mice were sacrificed to prepare single cell suspensions from draining lymph nodes or from spleen to assay ex vivo p33-specific cytotoxicity using ^{51}Cr-labeled EL-4 target cells pulsed with p33. Specific lysis of EL-4 target cells without p33 was less than 12% for all effectors. The results are shown in Figure 3.

Example 5

Dendritic Cells Purified from Mice Receiving Intrasplenic Injection of Peptide Potently Stimulate CTL

The effect of directing peptide delivery into lymphatic system was assessed. Peptide p33 was injected either i.v., s.c. or directly into the spleen of wild-type C57BL/6 mice. After 2 hours, DCs from the spleen of animals injected either i.s. or i.v., and additionally from the regional draining lymph nodes of animals injected s.c. Cells isolated from these tissues were sorted for DCs using magnetic beads coupled with a monoclonal antibody recognizing the integrin a chain, a marker specific for DCs in spleen and lymph nodes. The positively and the negatively sorted cell fractions were compared regarding their capacity to *in vitro* stimulate naive CTL from TCR transgenic mice specific for LCMV-gp. only when peptide had been directly injected into the spleen, the DC containing cell fraction stimulated CTL to proliferate. This indicated that CTL induction after direct injection of peptide into lymphatic organs reflected efficient loading of DCs with peptide. In contrast, the fraction depleted for DC did not induce proliferation and DCs isolated from lymphoid organs of i.v. and s.c injected mice were not effective stimulators. The results are shown in Figure 4.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

TABLE 1

C57BL/6 Mice	Virus Titer (Log_{10})
Single injection	4.2
Single injection	4.6
Single injection	4.0
Pump delivered	2.2
Pump delivered	1.8
Pump delivered	2.0
Unprimed	4.8
Unprimed	3.8
Unprimed	4.4

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I Claim:

1. A method of inducing a CTL response to an antigen comprising delivering the antigen into the lymphatic system of an animal.

2. A method according to claim 1, wherein the antigen is delivered continuously into the lymphatic system.

3. A method according to claim 1, wherein the antigen is delivered in a controlled release delivery vehicle.

4. A method according to claim 3, wherein the controlled release delivery vehicle is an osmotic pump.

5. A method according to claim 4, wherein the pump is implanted into the animal at or near a site of a lymphatic organ.

6. A method according to claim 5, wherein the pump is implanted near a lymph node.

7. A method according to claim 6 wherein the pump contains a means to deliver the antigen into the lymph node.

8. A method according to claim 4, wherein the pump is located external to the animal and comprises a means to deliver the antigen into the lymphatic system.

9. A method according to claim 8, wherein the means comprises a tube that is cannulated into a lymphatic vessel.

10. A method according to claim 3, wherein the controlled release delivery vehicle is a biodegradable microsphere.

11. A method according to claim 3, wherein the controlled release delivery vehicle is a liposome.

12. A method according to claim 1, wherein the antigen is a tumor antigen.

13. A method according to claim 12, wherein the antigen is selected from the group consisting of differentiation antigens, tumor-specific multilineage antigens, overexpressed embryonic antigens, overexpressed oncogenes, mutated tumor-suppressor genes, and viral antigens.

14. A method according to claim 12, wherein the antigen is selected from the group consisting of MART-1/MelanA (MART-1), gp100 (Pmel 17), tyrosinase, TRP-1, TRP-2, MAGE-1, MAGE-3, BAGE, GAGE-1, GAGE-2, p15 (58), CEA, p53, Ras, HER-2/neu, BCR-ABL, E2A-PRL, H4-RET, IGH-IGK, MYL-RAR, EBVA, (HPV) antigens E6 and E7.

15. A method according to claim 3, wherein the delivery vehicle further comprises a cytokine that is capable of enhancing the CTL response.

16. A method according to claim 15, wherein the cytokine is selected from the group consisting of GM-CSF, IL-12, IL-2, TNF and IFNγ.

17. A method according to claim 7 wherein the means to deliver the antigen is a catheter that is attached to the pump and inserted into a lymph node.

ABSTRACT OF THE DISCLOSURE

A method of inducing a cytotoxic T-lymphocyte (CTL) response to an antigen is disclosed. The method involves delivering the antigen directly into the lymphatic system of an animal. The antigen is preferably delivered using a controlled release delivery vehicle such as an osmotic pump. The method is advantageous over prior art methods for inducing a CTL response in that it does not require repetitive immunizations or the use of adjuvants. The method of the present invention can be used for the induction of CTLs in tumor immunotherapy.

Attorney Docket No. 7933.146US01

MERCHANT, GOULD, SMITH, EDELL, WELTER & SCHMIDT

United States Patent Application

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that

I verily believe I am the original, first and sole inventor (if only one name is listed below) or a joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: A METHOD OF INDUCING A CTL RESPONSE

The specification of which
a. ☒ is attached hereto
b. ☐ was filed on as application serial no. and was amended on (if applicable) (in the case of a PCT-filed application) described and claimed in international no. filed and as amended on (if any), which I have reviewed and for which I solicit a United States patent.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56 (attached hereto).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on the basis of which priority is claimed:

a. ☐ no such applications have been filed.
b. ☒ such applications have been filed as follows:

FOREIGN APPLICATION(S), IF ANY, CLAIMING PRIORITY UNDER 35 USC § 119			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)
Canada	2,209,815	10 July 1997	
ALL FOREIGN APPLICATION(S), IF ANY, FILED BEFORE THE PRIORITY APPLICATION(S)			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)

I hereby claim the benefit under Title 35, United States Code, § 120/365 of any United States and PCT international application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. APPLICATION NUMBER	DATE OF FILING (day, month, year)	STATUS (patented, pending, abandoned)

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below:

U.S. PROVISIONAL APPLICATION NUMBER	DATE OF FILING (Day, Month, Year)

I hereby appoint the following attorney(s) and/or patent agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith:

Albrecht, John W.	Reg. No. 40,481	Lacy, Paul E.	Reg. No. 38,946
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Beard, John L.	Reg. No. 27,612	Lasky, Michael B.	Reg. No. 29,555
Beck, Robert C.	Reg. No. 28,184	Lindquist, Timothy A.	Reg. No. 40,701
Berman, Charles	Reg. No. 29,249	Lynch, David W.	Reg. No. 36,204
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Cooper, Victor G.	Reg. No. 39,641	Pauly, Daniel M.	Reg. No. 40,123
Crawford, Robert	Reg. No. 32,122	Plunkett, Theodore	Reg. No. 37,209
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Dalglish, Leslie E.	Reg. No. 40,579	Reich, John C.	Reg. No. 37,703
Daulton, Julie R.	Reg. No. 36,414	Reiland, Earl D.	Reg. No. 25,767
DiPietro, Mark J.	Reg. No. 28,707	Rittmaster, Ted R.	Reg. No. 32,933
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Kowalchyk, Alan W.	Reg. No. 31,535	Xu, Min S.	Reg. No. 39,536
Kowalchyk, Katherine M.	Reg. No. 36,848		

I hereby authorize them to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/ organization who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct Merchant, Gould, Smith, Edell, Welter & Schmidt to the contrary.

Please direct all correspondence in this case to Merchant, Gould, Smith, Edell, Welter & Schmidt at the address indicated below:

Merchant, Gould, Smith, Edell,
Welter & Schmidt
3100 Norwest Center
90 South Seventh Street
Minneapolis, MN 55402-4131

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

2	**Full Name Of Inventor**	**Family Name** Kundig	**First Given Name** Thomas	**Second Given Name**
0	**Residence & Citizenship**	**City** Zurich	**State or Foreign Country** Switzerland	**Country of Citizenship** United States & Switzerland
1	**Post Office Address**	**Post Office Address** c/o University of Zurich Hospital; Dermatology Clinic; Gloriastrasse Str. 31	**City** Zurich	**State & Zip Code/Country** 8091 Switzerland
Signature of Inventor 201:			**Date:**	

§ 1.56 Duty to disclose information material to patentability.

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is canceled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§ 1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

(1) prior art cited in search reports of a foreign patent office in a counterpart application, and

(2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

(1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or

(2) It refutes, or is inconsistent with, a position the applicant takes in:

(i) Opposing an argument of unpatentability relied on by the Office, or

(ii) Asserting an argument of patentability.

A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability.

(c) Individuals associated with the filing or prosecution of a patent application within the meaning of this section are:

(1) Each inventor named in the application:

(2) Each attorney or agent who prepares or prosecutes the application; and

(3) Every other person who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application.

(d) Individuals other than the attorney, agent or inventor may comply with this section by disclosing information to the attorney, agent, or inventor.

FIGURE 1

Continuous Release of Peptides Using Osmotic Pump Induces Potent CTL response in C57BL/6 Mice

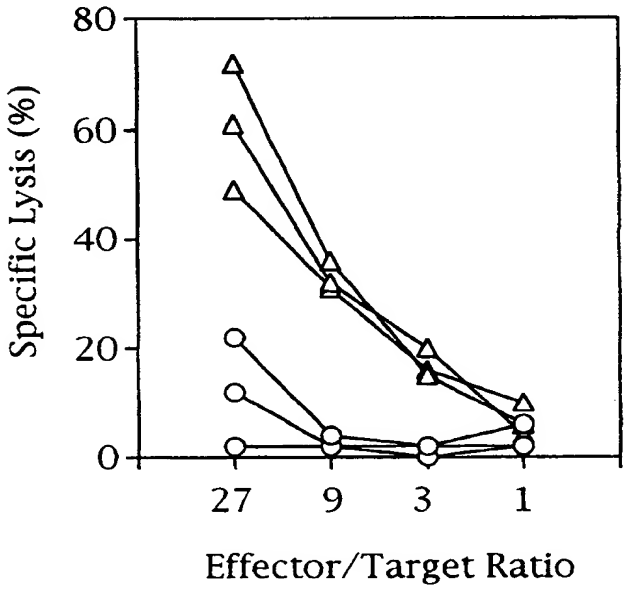

FIGURE 2

Continuous Release of Antigen Maintains
Potent CTL Effectors

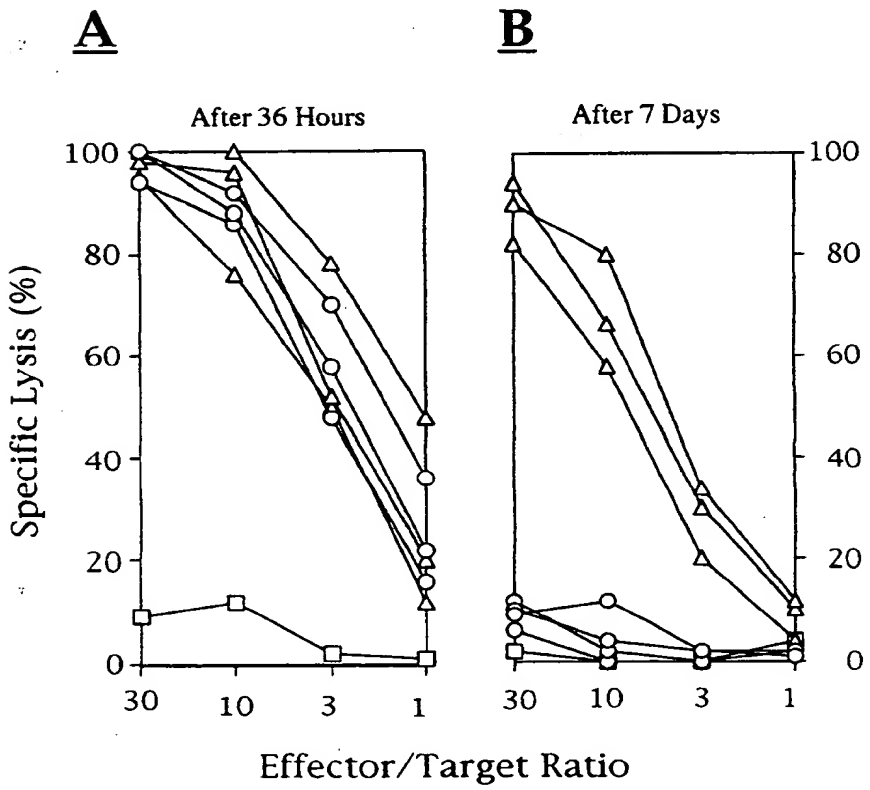

FIGURE 2 cont'd

Continuous Release of Antigen
Maintains Protective CTL Response
Against Virus Infection

C

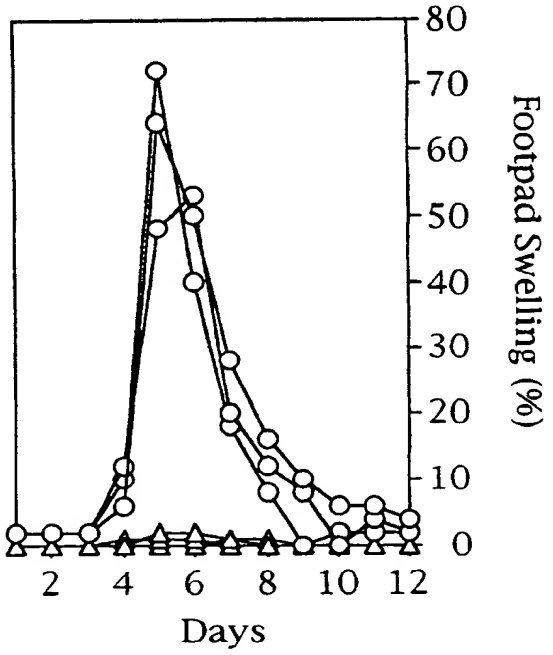

FIGURE 3

Direct delivery of antigen into lymphatic organ dramatically increases efficiency of CTL induction

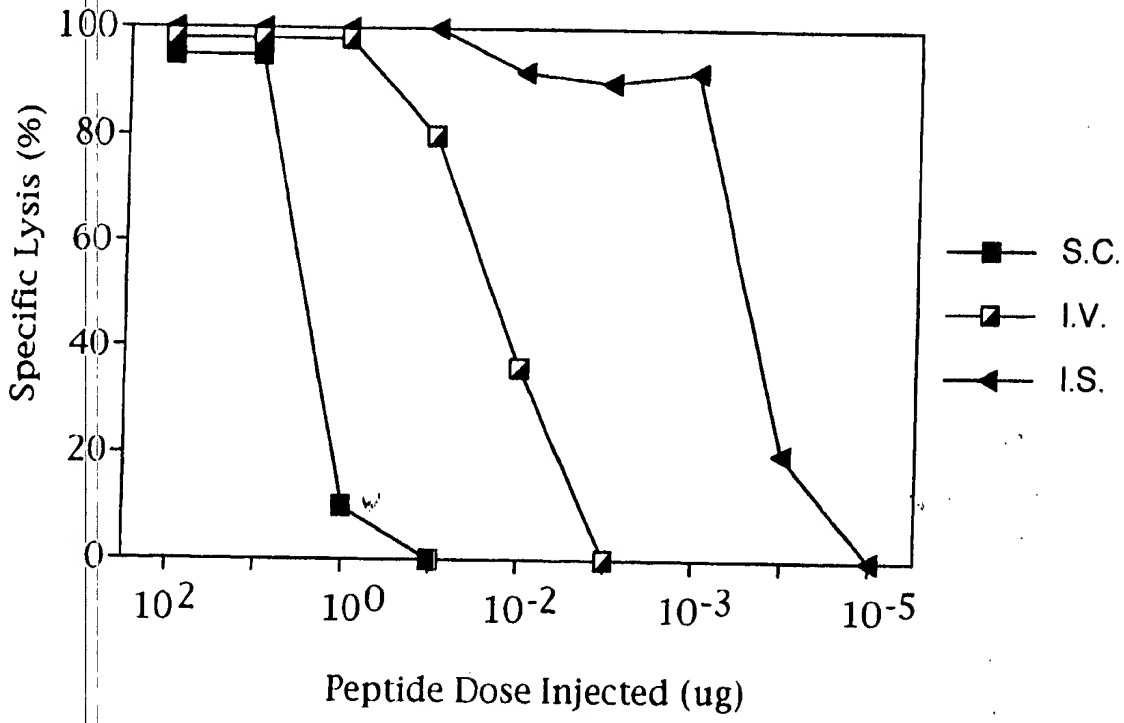

FIGURE 4

Dendritic Cells Purified from Mice Receiving Intrasplenic Injection of Peptide Potently Stimulate CTL